Aquatic Toxicology 211 (2019) 38–45

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Whether warming magnifies the toxicity of a pesticide is strongly dependent on the concentration and the null model

Vienna Delnat*, Lizanne Janssens, Robby Stoks

Laboratory of Evolutionary Stress Ecology and Ecotoxicology, University of Leuven, Belgium

ARTICLE INFO

Keywords:
Climate change
'Climate-induced toxicant sensitivity' (CITS) concept
Multiple stressors
Null model
Pesticide
Synergistic interaction

ABSTRACT

How global warming changes the toxicity of contaminants is a research priority at the intersection of global change biology and ecotoxicology. While many pesticides are more toxic at higher temperatures this is not always detected. We studied whether deviations from this general pattern can be explained by concentration-dependent interaction effects and by testing the interaction against the inappropriate null model. We exposed larvae of the mosquito *Culex pipiens* to three concentrations of the pesticide chlorpyrifos (absence, low and high) in the absence and presence of 4 °C warming. Both the low and high chlorpyrifos concentration were lethal and generated negative sublethal effects: activity of acetylcholinesterase (AChE) and total fat content decreased, and oxidative damage to lipids increased, yet growth rate increased. Warming was slightly lethal, yet had positive sublethal effects: growth rate, total fat content and metabolic rate increased, and oxidative damage decreased. For four out of seven response variables the independent action model identified the expected synergistic interaction between chlorpyrifos and warming. Notably, for three variables (survival, AChE and fat content) this was strongly dependent on the chlorpyrifos concentration, and for two of these (AChE and fat content) not associated with a significant interaction in the general(ized) linear models. For survival and fat content, warming only potentiated chlorpyrifos (CPF) toxicity at the low CPF concentration, while the opposite was true for AChE. Our results highlight that taking into account concentration-dependence and appropriate null model testing is crucial to improve our understanding of the toxicity of contaminants in a warming world.

1. Introduction

How stressors interact may determine the fate of populations and is important to prioritize conservation measures (Côté et al., 2016; Liess et al., 2016; Schäfer and Piggott, 2018). Nevertheless, and despite widespread co-exposure of organisms (Liess et al., 2016), combinations of contaminants and natural stressors are still largely ignored in ecological risk assessment (Goussen et al., 2016). The interaction type between stressors is determined relative to a null model that predicts the combined effect assuming the stressors are independent (Côté et al., 2016; Schäfer and Piggott, 2018). An interaction between stressors that results in a lesser combined effect than that predicted by a null model is an antagonism, while an interaction between stressors that results in a stronger combined effect of stressors than that predicted by a null model is a synergism (Côté et al., 2016; Schäfer and Piggott, 2018). Recent studies highlighted that general linear models may fail to identify the correct interaction type and the need to consider the appropriate null model to derive valid conclusions about the interaction type between stressors (Côté et al., 2016; Griffen et al., 2016; Schäfer and Piggott, 2018). For example, for the same magnitude of the combined response of two stressors the additive null model (used in general lineral models, GLMs) can falsely identify an antagonistic or additive effect, while the multiplicative null model (used in the independent action model, IA) can identify a synergism (Fig. 1). Nevertheless, the use of correct null model has been often ignored in the context of combinations of contaminants and natural stressors (but see e.g.Coors and De Meester, 2008; Korkaric et al., 2015; Pestana et al., 2009; Yu et al., 2015).

Abbreviations: AChE, acetylcholinesterase; CITS, climate-induced toxicant sensitivity'concept; CPF, chlorpyrifos; DTNB, dithiobis-2-nitro-benzoic acid; ETS, electron transport system; GLM, General Linear Model; GLMM, Generalized Linear Mixed Model; IA, Independent Action model; INT, p-iodonitrotetrazolium; IPCC, Intergovernmental Panel on Climate Change; L4, last larval instar; MDA, malondialdehyde; PBS-buffer, phosphate buffer saline; RCP, Representative Concentration Pathway; ROS, Reactive Oxygen Species; TBARS, thiobarbituric acid reactive substance; UPLC MS/MS, Ultra performance liquid chromatography - tandem mass spectrometer; WHO, World Health Organization

* Corresponding author at: Charles Deberiotstraat 32, box 2439, 3000 Leuven, Belgium.
E-mail address: vienna.delnat@kuleuven.be (V. Delnat).

https://doi.org/10.1016/j.aquatox.2019.03.010
Received 21 January 2019; Received in revised form 14 March 2019; Accepted 14 March 2019
Available online 20 March 2019
0166-445X/ © 2019 Elsevier B.V. All rights reserved.

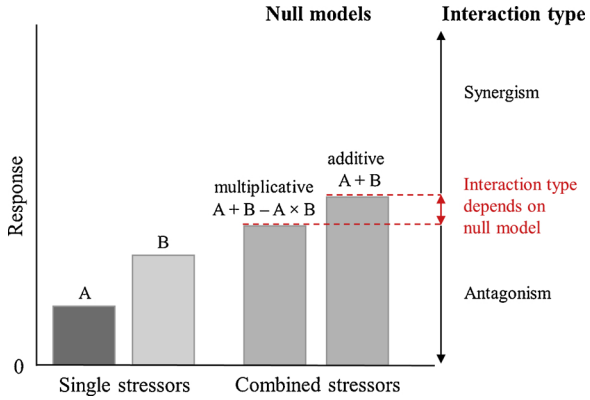

Fig. 1. Hypothetical scenario illustrating how identification of the interaction type between two stressors A and B depends on the null model against which the interaction is tested (adapted from Fig. 1 of Côté et al., 2016). The combined effect of stressors A and B can be additive (no interaction), synergistic (stronger effect than predicted by a null model) or antagonistic (weaker effect than predicted by a null model). In the range of combined responses indicated in red, the multiplicative null model used by the independent action (IA) model identifies a synergism, while the additive null model used by general linear models identifies an antagonism or an additive effect (at the upper limit of the red zone). Note that in the latter case the GLM would not detect a significant interaction while the IA model would detect a significant synergism.

Especially how global warming changes the toxicity of contaminants has been highlighted as a research priority (Van den Brink et al., 2018. The emerging general pattern of a higher toxicity of metals and many pesticides under warming (Heugens et al., 2001; Holmstrup et al., 2010has been formalized into the 'climate-induced toxicant sensitivity' concept (CITS; Moe et al., 2013; Noyes and Lema, 2015and challenges risk assessment of contaminants in a warming world (Landis et al., 2013; Moe et al., 2013; Noyes and Lema, 2015. Yet, there are empirical studies not supporting the concept even within groups of contaminants that typically show the predicted synergism (e.g. Kimberly and Salice, 2014; Perschbacher, 2005; Scheil and Köhler, 2009. Increasing our understanding of such deviations is crucial to improve risk assessment of contaminants in a warming world.

One possible reason for not identifying the expected synergism between a contaminant and warming is the misidentification of the interaction effect due to the usage of a wrong null model (Côté et al., 2016; Schäfer and Piggott, 2018). Given the mode of action of contaminants and warming is typically different, the multiplicative null model as implemented in the independent action model is recommended and not the additive model as implemented in standard general linear models (Schäfer and Piggott, 2018). As illustrated in Fig. 1, conclusions for the same combined stress response may match the expected synergism between contaminants and warming when using the appropriate multiplicative null model, yet may apparently deviate from the CITS concept when using the inappropriate additive null model.

A second possible reason of deviations of the CITS concept may be concentration dependence of the interaction between contaminants and warming. Despite the well-known pattern that interactions between contaminants may critically depend on the concentration (e.g. Maazouzi et al., 2016; Pacheco et al., 2018), this has been much less considered for interactions between a contaminant and a natural stressor (but see e.g. Korkaric et al., 2015; Yu et al., 2015). This is partly because the majority of multi-stressor studies only considered two levels per stressor (Griffen et al., 2016). Very few studies indeed tested the combined effects of multiple contaminant concentrations and temperature (but see e.g. Kimberly and Salice, 2014; Seeland et al., 2013; Vellinger et al., 2012). Moreover, to the best of our knowledge, none of these studies explicitly considered how the interaction type between the contaminant and warming can be concentration-dependent, thereby potentially causing a deviation from the CITS concept.

In this study we tested whether the interaction type between a pesticide and warming differed between a low and high pesticide concentration and whether this may cause deviations from the CITS concept. In addition, given that many tests of the CITS concept make conclusions based on significance of the contaminant-by-warming interaction in a general(ized) linear model, rather than the more appropriate independent action model (Schäfer and Piggott, 2018; Fig. 1), we evaluated whether this could generate apparent deviations from the CITS concept. Studies on the CITS concept typically focused on effects on life history (mainly mortality), yet additional insights may be gained by also evaluating effects on physiology (e.g. Op de Beeck et al., 2017b). Physiology may inform about the mechanisms underlying the patterns in life history, but also identify interaction types not captured by life history (e.g. Janssens and Stoks, 2013a; Karl et al., 2011). We therefore tested effects on both life history and physiology with as general CITS-based hypothesis that warming would magnify the negative effects of the pesticide (Heugens et al., 2001; Holmstrup et al., 2010; Noyes and Lema, 2015).

As pesticide, the organophosphate chlorpyrifos (CPF) was chosen because this is an important contaminant in aquatic systems. CPF has been identified as priority pollutant by the European Water Framework Directive (2000/60/EC), and is listed in the top ten chemicals that have the highest risk to aquatic organisms in UK surface waters (Johnson et al., 2017). CPF inhibits acetylcholinesterase, an important enzyme in the nervous system, thereby causing muscle spasms and eventually death (Eaton et al., 2008; Gupta, 2011). The Northern house mosquito *Culex pipiens* (Linaeus, 1758) form molestus was used as study species. Mosquitoes play an important role as prey species in both the aquatic and the terrestrial food webs (Becker et al., 2010), and therefore are important non-target species to be considered in ecological risk assessment of pesticides in temperate pond food webs (e.g. Rubach et al., 2012). Several studies showed that chlorpyrifos is more toxic at higher temperatures in aquatic insects (e.g. Dinh Van et al., 2014; Janssens and Stoks, 2013a, 2013b; Lydy et al., 1999), including the study species (Tran et al., 2018), making it especially relevant to study the impact of CPF under warming.

2. Materials and methods

2.1. Experimental design

We tested the single and combined effects of warming and pesticide exposure using a full factorial design with two temperature treatments (20 °C vs 24 °C) crossed with three pesticide treatments (solvent control, low CPF and high CPF). The rearing temperature of 20 °C represents the current mean summer water temperature of ponds in Germany where the mosquito culture originates (Tran et al., 2016), while 24 °C matches the expected mean temperature by 2100 under the 4 °C warming scenario RCP 8.5 (IPCC, 2013). The mosquito culture was maintained for more than 10 generations in the laboratory at ca. 20 °C and a light:dark period of 14:10 h prior to the experiment. The temperature treatment started in the egg stage, while animals were exposed to the pesticide for two days in the last larval instar (L4). Based on the guidelines by WHO (2005), larvae were exposed in the L4 stage in groups of 30 since this is the most resistant stage. We started 21–62 vials per treatment combination (total of 226 vials and 6780 mosquito larvae). More vials were started in the treatments with CPF, especially at high CPF, to obtain enough larvae that survived the exposure period for the measurements of physiology. Exact numbers of replicate vials per treatment combination are shown in Fig. 2A.

The mosquito larvae were kept until their molt in L4 in white 2 L containers (18.0 × 13.3 x 12.1 cm^3, made of polypropylene) filled with 1 L dechlorinated tap water. Containers were randomly allocated to a temperature treatment (20 °C or 24 °C) at a 14:10 h light:dark

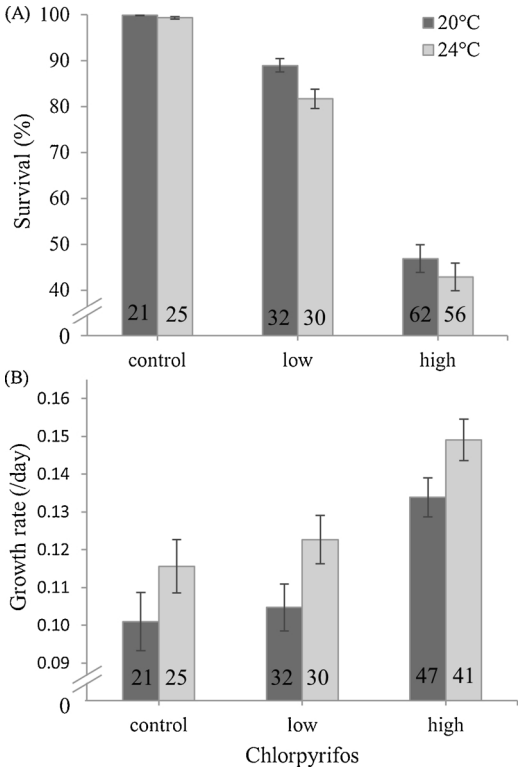

Fig. 2. Single and combined effects of the pesticide chlorpyrifos (CPF) and warming on life history variables of larvae of the mosquito *C. pipiens*: (A) survival, and (B) growth rate. Means are given ± 1 SE. Numbers inside the bars of the life history variables indicate the number of replicate vials (each vial contained 30 L4 larvae).

photoperiod in incubators (2 incubators per temperature). We kept a set of ca. 130 larvae (from 2 egg clutches) in each 2 L container. Larvae were fed daily with 1.6 mL of a 20 g/L mixture of Olvarit® 7 cereal flakes (46%), wheat germs (51%) and Supradyn® vitamins (3%). This equals 0.32 mg of food/day/larva which is ad libitum (Op de Beeck et al., 2016). The pesticide treatment was started within 16 h after larvae molted into the last larval instar (L4). At that moment, larvae were placed per 30 in 210 mL glass vials filled with 100 mL medium. Vials were randomly attributed to one of the three pesticide treatments. These vials were placed in the same incubators as the 2 L containers at a 14:10 h light:dark photoperiod and kept at the same temperature as before (20 °C or 24 °C). Larvae were counted daily during the two-day pesticide exposure period and we adjusted food rations to keep the food level constant per larva (at 0.32 mg of food/day/larvae) to avoid possible density effects. During the two-day pesticide exposure period we daily renewed the medium (after 24 h and after 48 h) to keep the CPF concentrations constant between temperature treatments. Two hours after the latest pesticide renewal, five L4 larvae per vial were placed on tissue paper to remove the water on the outer surface of the larvae. They were weighed to the nearest 0.01 mg using an electronic balance (AB135-S, Mettler Toledo), transferred to a single Eppendorf tube and immediately frozen using dry ice. The samples were stored at −80 °C till physiological analysis.

2.2. Pesticide concentrations

Chlorpyrifos (CPF, purity grade > 99%) was purchased at Sigma-Aldrich (St. Louis, Missouri, USA). Based on a range-finding experiment (see Appendix A) we selected two concentrations that gave ca. 15% (0.37 µg/L, 1.48 nmol/L) and ca 50% mortality (0.44 µg/L, 1.76 nmol/L) after 48 h with two exposure medium renewals (after 24 h and 48 h). These concentrations of CPF are ecologically relevant as they can be encountered in edge-to-field water bodies (Bernabò et al., 2011). The CPF solution was prepared by using a stock solution of 100 µg/mL CPF dissolved in absolute ethanol which was kept in the dark at 4 °C. From this stock solution, a second stock solution of 1 µg/mL was made in Milli-Q water. All pesticide treatments, including the solvent control, contained 3.7 µL ethanol/L. In a pilot experiment, ethanol concentrations up to 500 µL/L did not affect survival and growth of larvae of the study species (Tran et al., unpublished data).

During the two-day pesticide exposure period the pesticide medium was daily renewed. We took samples from 3 to 4 vials of the CPF treatments at the start and after 24 h (before the next renewal of the medium) on both temperatures. CPF concentrations were quantified at the Division of Soil and Water Management of the KU Leuven using UPLC MS/MS with Triple Quadrupole Mass Spectrometry. The initial low CPF concentration was 0.3925 ± 0.0248 µg/L (mean ± SE, $N = 4$ vials) and the initial high CPF concentration was 0.5458 ± 0.0266 ($N = 3$ vials). After 24 h at 20 °C, the low CPF concentration was 0.0953 ± 0.0120 µg/L and the high CPF concentration was 0.1022 ± 0.0283 µg/L ($N = 4$ vials). After 24 h at 24 °C, the low CPF concentration was 0.0903 ± 0.0153 µg/L and the high CPF concentration was 0.1061 ± 0.0092 µg/L ($N = 4$ vials).

2.3. Life history response variables

Survival during the two-day pesticide exposure period was scored as 1 (alive) and 0 (dead) for each larva within a vial. Growth rate was estimated per vial as the increase in body mass during the two-day pesticide exposure period. Growth rate was quantified as [ln(end mass)-ln(start mass)]/(2 days). Per vial, the start mass was set as the average mass of extra sets of five pooled larvae that did not enter the experiment, but had the same age as the larvae of the vial when they molted into the L4 stage. The end mass per vial was taken as the average mass after the two-day during pesticide exposure of two sets of five pooled larvae of that vial (or based on one set when not enough survivors were present in that vial).

2.4. Physiological response variables

Five physiological response variables were measured. Acetylcholinesterase (AChE), the target enzyme inhibited by organophosphates such as CPF (Domingues et al., 2010), was measured based on a modified protocol of Jensen et al. (1997). Total fat content, the major long-term energy storage in insects (Azeez et al., 2014), was quantified based on a modified protocol of Marsh and Weinstein (1966). The activity of the mitochondrial electron transport system (ETS), an estimate of metabolic rate, was determined based on the modified protocol of De Coen and Janssen (1997). Finally, two physiological variables related to oxidative stress were quantified. The concentration of the superoxide anion (O_2^-), a highly toxic reactive oxygen species, was measured based on the protocol of Oracz et al. (2007). As a measure of oxidative damage to lipids, the level of malondialdehyde (MDA) was determined based on a modified protocol of Miyamoto et al. (2011). A detailed description of the protocols can be found in Appendix B.

2.5. Statistical analyses

All statistical analyses were performed in R v3.3.2. (R Core Team, 2017) with the packages lme4 v1.1–13 (Bates et al., 2015), car v2.1–5 (Fox and Weisberg, 2002), afex v0.18-0 (Singmann et al., 2017) and lsmeans v2.26-3 (Lenth, 2016).

We tested for effects of temperature, chlorpyrifos (CPF) and their interaction on individual survival (alive/dead) using a generalized linear mixed model with a binomial error structure and the logit link. To take into account that animals from the same vial were not independent we added vial to the model as a random factor.

Table 1
Results of the general(ized) linear (mixed) models testing for the effects of temperature and exposure to chlorpyrifos (CPF) on two life history variables and five physiological variables of larvae of the mosquito *Culex pipiens*. The *P*-values indicated in bold are significant ($P < 0.05$). Df is the abbreviation of the degree of freedom.

	Survival			Growth rate		
	χ^2-value	Df	*P*-value	*F*-value	Df	*P*-value
Temperature	6.895	1	**0.0086**	9.40	1, 190	**0.0025**
CPF	733.576	2	**< 0.001**	18.03	2, 190	**< 0.001**
Temperature × CPF	11.985	2	**0.0025**	0.039	2, 190	0.96
	AChE activity			Total fat		
	F-value	Df	*P*-value	*F*-value	Df	*P*-value
Temperature	1.21	1	0.27	59.73	1, 84	**< 0.001**
CPF	5.53	2	**0.0055**	6.26	2, 84	**0.0029**
Temperature × CPF	0.61	2	0.54	0.27	2, 84	0.77
	ETS activity			Superoxide anion conc.		
	F-value	Df	*P*-value	*F*-value	Df	*P*-value
Temperature	0.88	1	0.35	0.003	1, 84	0.96
CPF	8.40	2	**< 0.001**	1.69	2, 84	0.19
Temperature × CPF	3.47	2	**0.036**	0.33	2, 84	0.72
	MDA level					
	F-value		Df			*P*-value
Temperature	18.35		1			**< 0.001**
CPF	7.07		2			**0.00146**
Temperature × CPF	0.010		2			0.99

To test if temperature, CPF and their interaction had an effect on growth rate and the five physiological response variables, we used general linear models (GLM) with a normal error structure and the identity link. To meet the model assumptions, the total fat content, the superoxide anion concentration and the AChE activity were ln-transformed. When there was a significant effect of the CPF treatment (which had three levels) or a significant interaction between temperature and CPF, we performed Tukey HSD post hoc tests to explore which treatment levels differed.

To formally identify the interaction type (additive, synergism or antagonism) between the two stressors with a different mode of action we used the independent action (IA) model as recommended by Schäfer and Piggott (2018). Furthermore, the IA model takes explicitly into account that a given individual killed by one stressor can no longer be killed by the other stressor (see Table 1 in Schäfer and Piggott, 2018). We determined the interaction type separately for the low and high CPF concentrations given that the interaction type might be concentration-dependent. We applied the IA model following the procedure of Coors and De Meester (2008). For details of this procedure, see Appendix C.

3. Results

3.1. Life history

Overall, warming reduced survival but this was much less pronounced in the solvent control (Tukey: $P = 0.052$) than at low and high CPF (Tukey: both $P \leq 0.032$) (Temperature and Temperature × CPF, Table 1, Fig. 2A). CPF exposure reduced survival with ca. 15% at low CPF (Tukey: $P < 0.001$) and with ca. 50% at high CPF (Tukey: $P < 0.001$) (CPF, Table 1). Warming increased the CPF-induced mortality only at low CPF (Tukey: $P < 0.001$) but not at high CPF (Tukey: $P = 0.32$) (Temperature × CPF, Table 1). The IA model indicated that warming and CPF interacted synergistically at low CPF, but additively at high CPF (Appendix D Table D.1 A).

The growth rate was higher under warming and higher when exposed to high CPF (Tukey: $P < 0.001$), but not when exposed to low CPF (Tukey: $P = 0.71$) (Temperature and CPF, Table 1, Fig. 2B). There was no significant interaction for growth between temperature and CPF (Temperature × CPF, Table 1). Also the IA model identified the interaction between CPF and temperature as additive for both low and high CPF (Appendix D Table D.1B).

3.2. Physiology

Warming did not affect the activity of acetylcholinesterase (AChE) (Temperature, Table 1, Fig. 3A). Overall, CPF exposure resulted in a lower AChE activity (CPF, Table 1), this was only marginally significant at low CPF (Tukey: $P = 0.060$) and more obvious at high CPF (Tukey: $P = 0.005$)(Fig. 3A). There was no significant Temperature × CPF interaction in the GLM (Table 1). While the IA model indicated an additive effect between temperature and low CPF, it identified a synergistic interaction between temperature and high CPF (reflecting a stronger CPF-induced reduction in AChE at 24 °C than at 20 °C) (Appendix D Table D.1C, Fig. 3A).

Warming increased the total fat content (Temperature, Table 1, Fig. 3B). Instead, exposure to both low and high CPF reduced the total fat content (CPF, Table 1, Fig. 3B, Tukey: both $P \leq 0.023$). The Temperature × CPF interaction was not significant (Table 1). However, the IA model indicated a synergistic interaction between temperature and low CPF (reflecting a stronger CPF-induced reduction in fat content at 24 °C than at 20 °C), yet an additive effect at high CPF (Appendix D Table D.1D, Fig. 3B).

Warming increased the activity of the electron transport system (ETS) only in the solvent control (Tukey: $P = 0.042$) but not in the presence of CPF (Tukey: both $P \geq 0.52$) (Temperature × CPF, Table 1, Fig. 3C). Both low and high CPF exposure reduced ETS activity, but only at 24 °C (Tukey: both $P \leq 0.049$) and not at 20°C (Tukey: both $P \geq 0.41$) (Temperature × CPF, Table 1). The IA model showed a

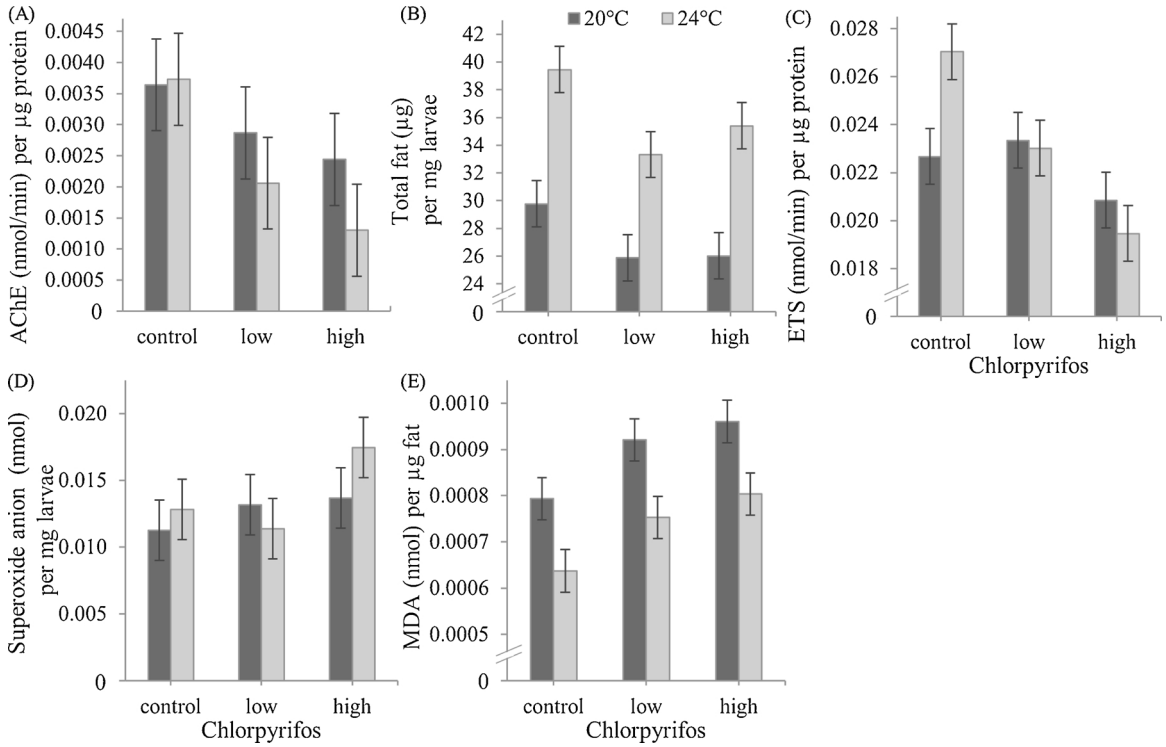

Fig. 3. Single and combined effects of the pesticide chlorpyrifos (CPF) and warming on physiological variables of larvae of the mosquito *C. pipiens*: (A) acetylcholinesterase (AChE) activity, (B) total fat content, (C) ETS activity, (D) superoxide anion concentration, and (E) MDA level. Means are given ± 1 SE. The physiological variables are based on 15 replicate vials.

synergistic interaction for both low and high CPF (reflecting a stronger CPF-induced reduction in ETS at 24 °C than at 20 °C; Appendix D Table D.1E, Fig. 3C).

Neither the effects of warming and CPF nor their interaction were significant for the superoxide anion concentration (Table 1, Fig. 3D). Accordingly, the IA model identified an additive effect between temperature and CPF at both CPF concentrations (Appendix D Table D.1 F).

Warming reduced the malondialdehyde (MDA) level (Temperature, Table 1, Fig. 3E). Instead, exposure to both low and high CPF increased MDA (CPF, Table 1, Tukey: both $P \leq 0.026$). There was no significant Temperature × CPF interaction (Table 1). The IA model identified an additive effect between temperature and CPF at both CPF concentrations (Appendix D Table D.1 G).

4. Discussion

Warming and especially chlorpyrifos (CPF) were lethal as stressors. In the absence of warming, CPF in general also was stressful by generating negative sublethal effects in the survivors: it decreased AChE activity and total fat content, and increased oxidative damage to lipids (measured as MDA), yet it increased growth rate. In the absence of the pesticide, warming had positive sublethal effects in the survivors: it increased growth rate, total fat content and ETS activity, and decreased the MDA level. We found partial support for the CITS concept. For five out of seven response variables the independent action (IA) model identified the expected synergistic interaction between CPF and warming. Yet, not for all variables and three of the identified synergistic interactions were dependent on the concentration of CPF, and not always associated with a significant interaction in the linear models.

4.1. Effects of warming in the absence of the pesticide

A temperature increase of 4 °C, as expected by 2100 under IPCC (2013) scenario RCP8.5, slightly reduced the survival of *C. pipiens*. This is consistent with previous results on thermal adaptation in the study species since 20 °C corresponds with the mean summer water temperatures of the field sites in Germany where the lab culture originates from (Tran et al., 2018, 2016). In contrast, in the absence of the pesticide warming had positive (associated with presumably higher fitness values) effects in the survivors by increasing growth rate, total fat content and ETS activity, and decreasing the MDA level. The increase in growth rate under warming might be an adaptive strategy to shorten the life cycle. This suggests that despite 24 °C being slightly lethal, this temperature was closer to the optimal temperature for growth than 20 °C. This reflects the general pattern that many temperate ectotherms have a thermal optimum that is higher than the mean ambient temperature (Deutsch et al., 2008). Moreover, thermal optima may differ between traits (here survival vs growth rate) (Sinclair et al., 2016). The higher growth rate at 24 °C may on its turn explain the higher metabolic activity (measured as ETS), as growth rate and metabolic rate are often positively coupled (Downs et al., 2016). An increase in metabolic rate can cause increases in reactive oxygen species (ROS) and oxidative damage (Lushchak, 2011), yet in our study warming did not increase the superoxide anion concentration and even reduced the level of MDA. In larvae of the damselfly *Enallagma cyathigerum* warming did also not increase levels of ROS and oxidative damage which could be explained by the observed upregulation of two antioxidant enzymes (Janssens and Stoks, 2017; see also Tu et al., 2018). Possibly, in our study warming created an over-compensatory antioxidant response resulting in lower MDA levels (see e.g. Costantini et al., 2010; Sohal and Weindruch, 1996).

4.2. Effects of chlorpyrifos in the absence of warming

Survival at 20 °C decreased with ca. 15% when exposed to low CPF (measured concentration = 0.39 µg/L) and with ca. 50% when exposed to high CPF (measured concentration = 0.55 µg/L). The here documented $LC_{50,48h}$ is more than two times higher than the 0.2 µg/L

reported for *C. pipiens* by Rubach et al. (2012). CPF also was stressful by generating negative sublethal effects in the survivors: it decreased AChE activity and total fat content, and increased oxidative damage to lipids (measured as MDA). These sublethal effects indicate that the survivors will likely still suffer negative post-exposure effects on fitness. For example, lower lipid levels in *Culex* sp. have been associated with delayed oogenesis (Shin et al., 2012), and a decreased female survival (Vrzal et al., 2010). Furthermore, a higher oxidative damage to lipids has been shown to cause a shorter adult lifespan in another semi-aquatic insect (Janssens and Stoks, 2018). The only positive effect of CPF exposure was the increased growth rate at the high CPF concentration, which may indicate an escape response toward the terrestrial adult stage. Similar escape responses where semi-aquatic insects accelerated growth and development to avoid aquatic exposure to CPF have been documented in the study species (Delnat et al., 2019), and in other taxa such as damselfly larvae (Janssens and Stoks, 2013b) and fiddler crabs (Weis and Mantel, 1976). However, note that this increased growth rate did not occur at a low CPF concentration which can be explained on the one hand by the magnitude of the stressor not being high enough to cause this escape response or on the other hand that survival selection retained only survivors of high quality. Consistent with its mode of action (Eaton et al., 2008), under CPF exposure AChE was inhibited. This, together with the CPF-induced increase in oxidative damage (measured as MDA), may have contributed to the increased mortality. Pesticides such as CPF are known to increase oxidative damage, for example in fish (Zahran et al., 2018) and in semi-aquatic insects (Janssens and Stoks, 2017; Op de Beeck et al., 2017a). The increase in oxidative damage in CPF-exposed larvae could not be explained by higher superoxide anion levels as these remained constant. The latter is in contrast with the well-documented increase in ROS production in CPF-exposed animals (e.g. Cacciatore et al., 2015; Itziou et al., 2011; Jin et al., 2015; Patetsini et al., 2013). Possibly, the CPF-induced increase in MDA levels was due to an increase in other ROS (such as hydrogen peroxide and hydroxyl radicals). Moreover, even without an increase in ROS levels, CPF-induced reductions in the activity levels of antioxidant enzymes (e.g. Janssens and Stoks, 2017; Marigoudar et al., 2018), may have shifted the balance between ROS and antioxidant defense toward a state of oxidative stress resulting in an increase in MDA. Finally, CPF exposure caused a reduced energy storage as measured by a lowered total fat content (see e.g. also Arambourou and Stoks, 2015; Dinh Van et al., 2016). This might be explained by a reallocation of energy to detoxification processes (Campero et al., 2007).

4.3. Interactive effects between chlorpyrifos and warming

For four response variables (survival, ETS, fat content and AChE) the IA model identified a synergistic interaction indicating CPF being more toxic under warming. This matches the general CITS pattern that many pesticides, including organophosphates, are more toxic at higher temperatures (Hooper et al., 2013; Moe et al., 2013; Noyes and Lema, 2015). This confirms previous studies on aquatic animals such as fish (Patra et al., 2015; Philippe et al., 2018), damselfly larvae (Dinh Van et al., 2014) and the study species (Tran et al., 2018). This has been explained by a higher uptake and, specifically for CPF, also a faster conversion to the more toxic oxon-metabolite at higher temperatures (Buchwalter et al., 2003; Lydy et al., 1999).

We could demonstrate that whether the interaction between CPF and warming was synergistic depended for three variables (survival, AChE activity and fat content) on the CPF concentration. Notably, for survival and total fat content, warming only potentiated CPF toxicity at the low and not at the high CPF concentration. This switch from a synergistic to an additive interaction with increasing CPF concentration is intriguing. Likely, because of the considerably higher mortality at the high (ca. 50%) compared to at the low (ca. 15%) CPF concentration, more of the weakest larvae were already removed by the single exposure to high CPF, so that the additional warming stress had little extra effect on survival and fat content (see also Côté et al., 2016; Vinebrooke et al., 2004). Yet, for AChE the opposite switch occurred: from an additive interaction between low CPF and warming toward a synergistic interaction between high CPF and warming. To become toxic, chlorpyrifos needs to be bioactivated through transformation by enzymes into the chlorpyrifos-oxon metabolite that permanently inhibits AChE (Eaton et al., 2008; Flaskos, 2012). The combination of a higher biotransformation at higher temperatures and the presence of more CPF that can be converted to the metabolite at the high CPF concentration apparently resulted in a more than additive increase in the chlorpyrifos-oxon, hence inhibition of AChE. Note that the physiological measurements (including AChE) and growth could only be measured in survivors which could explain discrepancies between the patterns of survival and those of the other end points since these survivors showed a higher tolerance against CPF. Whatever the underlying reasons, our results indicate that the presence of a synergistic interaction between a pesticide and warming may not be general and strongly depend on the pesticide concentration. Note that even though the difference between both concentrations is small, they caused a considerable difference in survival (42%) in this study in this study reflecting the steepness of the dose-response curve (see Appendix A). This concentration-dependence is important as it may explain differences between studies in whether warming magnifies the toxicity of pesticides or not. Such concentration-dependent interaction effects have been observed before when contaminants were combined with other natural stressors. For example, there was an additive effect on mortality in the African clawed frog (*Xenopus laevis*) between UVB radiation and low endosulfan concentrations, yet at the highest endosulfan concentration the interaction became antagonistic (Yu et al., 2015).

Notably, for two variables (AChE activity and fat content) a synergistic interaction type was detected by the IA model while there was no significant Temperature × CPF interaction in the linear models (both $P > 0.50$). This can be explained because the IA model and the general linear models (GLMs) differ in their null model against which an interaction is tested (Schäfer and Piggott, 2018). As visualized in Fig. 1, the additive null model used in general lineral models indeed may detect an additive effect, while the multiplicative null model used in the independent action model (IA) detects a synergistic interaction effect (Côté et al., 2016). Similar to our results, Yu et al. (2015) also found no interaction effect between UVB radiation and α-cypermethrin for larval size of the African clawed frog based on the GLM, while the IA model identified a synergistic interaction effect between both stressors. Two alternative reasons for a mismatch between the GLMs and the IA model can be excluded in our study. First, the ln-transformation of both variables, which may affect significance of interaction terms in GLMs, did not play a role. Also for the non-transformed variables no significant interaction effect was found in the GLMs (both $P \geq 0.54$). Second, for both variables the synergism was only detected at one CPF concentration, which may have made it less likely to detect an overall interaction in the GLMs. Yet, also in separate GLMs per concentration, no significant temperature × CPF interactions were present (for AChE: both $P \geq 0.28$; for fat content: both $P > 0.58$). While there is a long tradition to explicitly use the IA model to detect and identify interactions between contaminants with a different mode of action (Schäfer and Piggott, 2018) this has been largely ignored when analyzing the combinations of a contaminant with a natural stressor (but see e.g. Coors and De Meester, 2008; Korkaric et al., 2015; Yu et al., 2015). Our results demonstrate that not explicitly applying the IA model may result in the failure to detect synergisms between a pesticide and warming.

5. Conclusions

Our study provides partial support for the CITS concept, and more importantly it identified two important, likely widespread reasons that may cause (apparent) deviations. First, we provided proof-of-principle that the synergistic interaction between temperature and CPF can be

concentration-dependent. Both concentrations we used caused mortality, integrating also non-lethal concentrations in future experiments would be rewarding by extending the range of concentrations toward levels where no survival selection occurs. Second, we demonstrated that the often used approach to test the CITS concept based on the significance of the contaminant-by-warming interaction in a GLM may be misleading, thereby supporting the recent and more general call to use appropriate null models when testing interactions between stressors (Schäfer and Piggott, 2018). Our results highlight that taking into account concentration-dependence and appropriate null model testing is crucial to improve our understanding of the toxicity of contaminants in a warming world.

Declarations of interest

None.

Funding

This work was supported by research grants from the KU Leuven (C16/17/002) and from the Fund for Scientific Research Flanders (FWO) (G.0524.17).

Acknowledgments

We thank Ria Van Houdt and Ian De Ridder for their assistance during the experiment and Ralf Schäfer for discussion of the different null models. VD is a PhD fellow of the Fund for Scientific Research Flanders (FWO).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2019.03.010.

References

Arambourou, H., Stoks, R., 2015. Combined effects of larval exposure to a heat wave and chlorpyrifos in northern and southern populations of the damselfly Ischnura elegans. Chemosphere 128, 148–154. https://doi.org/10.1016/j.chemosphere.2015.01.044.

Azeez, O.I., Meintjes, R., Chamunorwa, J.P., 2014. Fat body, fat pad and adipose tissues in invertebrates and vertebrates: the nexus. Lipids Health Dis. 13, 1–13. https://doi.org/10.1186/1476-511X-13-71.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67, 1–48. https://doi.org/10.18637/jss.v067.i01.

Becker, N., Petrić, D., Zgomba, M., Boase, C., Madon, M., Dahl, C., Kaiser, A., 2010. Mosquitoes and Their Control, second. ed. Springer, Verlag Berlin Heidelberg. https://doi.org/10.1007/978-3-540-92874-4.

Bernabò, I., Sperone, E., Tripepi, S., Brunelli, E., 2011. Toxicity of chlorpyrifos to larval *Rana dalmatina*: acute and chronic effects on survival, development, growth and gill apparatus. Arch. Environ. Contam. Toxicol. 61, 704–718. https://doi.org/10.1007/s00244-011-9655-1.

Buchwalter, D.B., Jenkins, J.J., Curtis, L.R., 2003. Temperature influences on water permeability and chlorpyrifos uptake in aquatic insects with differing respiratory strategies. Environ. Toxicol. Chem. 22, 2806–2812. https://doi.org/10.1897/02-350.

Cacciatore, L.C., Nemirovsky, S.I., Verrengia Guerrero, N.R., Cochón, A.C., 2015. Azinphos-methyl and chlorpyrifos, alone or in a binary mixture, produce oxidative stress and lipid peroxidation in the freshwater gastropod Planorbarius corneus. Aquat. Toxicol. 167, 12–19. https://doi.org/10.1016/j.aquatox.2015.07.009.

Campero, M., Slos, S., Ollevier, F., Stoks, R., 2007. Sublethal pesticide concentrations and predation jointly shape life history: behavioral and physiological mechanisms. Ecol. Appl. 17, 2111–2122. https://doi.org/10.1890/07-0442.1.

Coors, A., De Meester, L., 2008. Synergistic, antagonistic and additive effects of multiple stressors: predation threat, parasitism and pesticide exposure in *Daphnia magna*. J. Appl. Ecol. 45, 1820–1828. https://doi.org/10.1111/j.1365-2664.2007.0.

Costantini, D., Metcalfe, N.B., Monaghan, P., 2010. Ecological processes in a hormetic framework. Ecol. Lett. 13, 1435–1447. https://doi.org/10.1111/j.1461-0248.2010.01531.x.

Côté, I.M., Darling, E.S., Brown, C.J., 2016. Interactions among ecosystem stressors and their importance in conservation. Proc. R. Soc. B Biol. Sci. 283https://doi.org/10.1098/rspb.2015.2592. 20152592.

De Coen, W.M., Janssens, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV. Cellular Energy Allocation: a newmethodology to assess the energy budget of toxicant-stressed *Daphnia* populations. J. Aquat. Ecosyst. Stress Recover. 6, 43–55. https://doi.org/10.1023/A:1008228517955.

Delnat, V., Tran, T.T., Janssens, L., Stoks, R., 2019. Daily temperature variation magnifies the toxicity of a mixture consisting of a chemical pesticide and a biopesticide in a vector mosquito. Sci. Total Environ. 659, 33–40. https://doi.org/10.1016/J.SCITOTENV.2018.12.332.

Deutsch, C.A., Tewksbury, J.J., Huey, R.B., Sheldon, K.S., Ghalambor, C.K., Haak, D.C., Martin, P.R., 2008. Impacts of climate warming on terrestrial ectotherms across latitude. Proc. Natl. Acad. Sci. 105, 6668–6672. https://doi.org/10.1073/pnas.0709472105.

Dinh Van, K., Janssens, L., Debecker, S., Stoks, R., 2014. Temperature- and latitude-specific individual growth rates shape the vulnerability of damselfly larvae to a widespread pesticide. J. Appl. Ecol. 51, 919–928. https://doi.org/10.1111/1365-2664.12269.

Dinh Van, K., Janssens, L., Stoks, R., 2016. Exposure to a heat wave under food limitation makes an agricultural insecticide lethal: a mechanistic laboratory experiment. Glob. Change Biol. 22, 3361–3372. https://doi.org/10.1111/gcb.13415.

Domingues, I., Agra, A.R., Monaghan, K., Soares, A.M.V., Nogueira, A.J., 2010. Cholinesterase and glutathione-S-transferase activities in freshwater invertebrates as biomarkers to assess pesticide contamination. Environ. Toxicol. Chem. 29, 5–18. https://doi.org/10.1002/etc.23.

Downs, C.J., Brown, J.L., Wone, B.W.M., Donovan, E.R., Hayes, J.P., 2016. Speeding up growth: selection for mass-independent maximal metabolic rate alters growth rates. Am. Nat. 187, 295–307. https://doi.org/10.1086/684837.

Eaton, D.L.L., Daroff, R.B.B., Autrup, H., Bridges, J., Buffler, P., Costa, L.G.G., Coyle, J., McKhann, G., Mobley, W.C.C., Nadel, L., Neubert, D., Schulte-Hermann, R., Spencer, P.S.S., 2008. Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. Crit. Rev. Toxicol. 38, 1–125. https://doi.org/10.1080/10408440802272158.

Flaskos, J., 2012. The developmental neurotoxicity of organophosphorus insecticides: a direct role for the oxon metabolites. Toxicol. Lett. 209, 86–93. https://doi.org/10.1016/j.toxlet.2011.11.026.

Fox, J., Weisberg, S., 2002. An {R} Companion to Applied Regression, second. ed. Sage Publications. SAGE Publications, Inc, Thousand Oaks CA. https://doi.org/10.1177/0049124105277200.

Goussen, B., Price, O.R., Rendal, C., Ashauer, R., 2016. Integrated presentation of ecological risk from multiple stressors. Sci. Rep. 6, 1–11. https://doi.org/10.1038/srep36004.

Griffen, B.D., Belgrad, B.A., Cannizzo, Z.J., Knotts, E.R., Hancock, E.R., 2016. Rethinking our approach to multiple stressor studies in marine environments. Mar. Ecol. Prog. Ser. 543, 273–281. https://doi.org/10.3354/meps11595.

Gupta, R.C., 2011. Toxicology of Organophosphate and Carbamate Compounds. Academic Press.

Heugens, E.H.W., Hendriks, A.J., Dekker, T., Van Straalen, N.M., Admiraal, W., 2001. A review of the effects of multiple stressors on aquatic organisms and analysis of uncertainty factors for use in risk assessment. Crit. Rev. Toxicol. 31, 247–284. https://doi.org/10.1080/20014091111695.

Holmstrup, M., Bindesbøl, A.M., Oostingh, G.J., Duschl, A., Scheil, V., Köhler, H.R., Loureiro, S., Soares, A.M.V.M., Ferreira, A.L.G., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P.E., Bayley, M., Svendsen, C., Spurgeon, D.J., 2010. Interactions between effects of environmental chemicals and natural stressors: a review. Sci. Total Environ. 408, 3746–3762. https://doi.org/10.1016/j.scitotenv.2009.10.067.

Hooper, M.J., Ankley, G.T., Cristol, D.A., Maryoung, L.A., Noyes, P.D., Pinkerton, K.E., 2013. Interactions between chemical and climate stressors: a role for mechanistic toxicology in assessing climate change risks. Environ. Toxicol. Chem. 32, 32–48. https://doi.org/10.1002/etc.2043.

IPCC, 2013. Climate Change 2013: the Physical Science Basis. Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA. https://doi.org/10.1017/cbo9781107415324.

Itziou, A., Kaloyianni, M., Dimitriadis, V.K., 2011. Effects of organic contaminants in reactive oxygen species, protein carbonylation and DNA damage on digestive gland and haemolymph of land snails. Chemosphere 85, 1101–1107. https://doi.org/10.1016/j.chemosphere.2011.07.043.

Janssens, L., Stoks, R., 2013a. Synergistic effects between pesticide stress and predator cues: conflicting results from life history and physiology in the damselfly *Enallagma cyathigerum*. Aquat. Toxicol. 132–133, 92–99. https://doi.org/10.1016/j.aquatox.2013.02.003.

Janssens, L., Stoks, R., 2013b. Fitness effects of chlorpyrifos in the damselfly *Enallagma cyathigerum* strongly depend upon temperature and food level and can bridge metamorphosis. PLoS One 8https://doi.org/10.1371/journal.pone.0068107. e68107.

Janssens, L., Stoks, R., 2017. Chlorpyrifos-induced oxidative damage is reduced under warming and predation risk: explaining antagonistic interactions with a pesticide. Environ. Pollut. 226, 79–88. https://doi.org/10.1016/j.envpol.2017.04.012.

Janssens, L., Stoks, R., 2018. Rapid larval development under time stress reduces adult life span through increasing oxidative damage. Funct. Ecol. 32, 1036–1045. https://doi.org/10.1111/1365-2435.13068.

Jensen, C.S., Garsdal, L., Baatrup, E., 1997. Acetylcholinesterase inhibition and altered locomotor behavior in the carabid beetle *Pterostichus cupreus*. A linkage between biomarkers at two levels of biological complexity. Environ. Toxicol. Chem. 16, 1727–1732. https://doi.org/10.1002/etc.5620160822.

Jin, Y., Liu, Z., Peng, T., Fu, Z., 2015. The toxicity of chlorpyrifos on the early life stage of zebrafish: a survey on the endpoints at development, locomotor behavior, oxidative stress and immunotoxicity. Fish Shellfish Immunol. 43, 405–414. https://doi.org/10.1016/j.fsi.2015.01.010.

Johnson, A.C., Donnachie, R.L., Sumpter, J.P., Jürgens, M.D., Moeckel, C., Pereira, M.G., 2017. An alternative approach to risk rank chemicals on the threat they pose to the

aquatic environment. Sci. Total Environ. 599–600, 1372–1381. https://doi.org/10.1016/j.scitotenv.2017.05.039.
Karl, I., Stoks, R., De Block, M., Janowitz, S.A., Fischer, K., 2011. Temperature extremes and butterfly fitness: conflicting evidence from life history and immune function. Glob. Change Biol. 17, 676–687. https://doi.org/10.1111/j.1365-2486.2010.02277.x.
Kimberly, D.A., Salice, C.J., 2014. Complex interactions between climate change and toxicants: evidence that temperature variability increases sensitivity to cadmium. Ecotoxicology 23, 809–817. https://doi.org/10.1007/s10646-014-1221-y.
Korkaric, M., Behra, R., Fischer, B.B., Junghans, M., Eggen, R.I.L., 2015. Multiple stressor effects in *Chlamydomonas reinhardtii* - toward understanding mechanisms of interaction between effects of ultraviolet radiation and chemical pollutants. Aquat. Toxicol. 162, 18–28. https://doi.org/10.1016/j.aquatox.2015.03.001.
Landis, W.G., Durda, J.L., Brooks, M.L., Chapman, P.M., Menzie, C.A., Stahl, R.G., Stauber, J.L., 2013. Ecological risk assessment in the context of global climate change. Environ. Toxicol. Chem. 32, 79–92. https://doi.org/10.1002/etc.2047.
Lenth, R.V., 2016. Least-squares means: the r package lsmeans. J. Stat. Softw. 69, 1–33. https://doi.org/10.18637/jss.v069.i01.
Liess, M., Foit, K., Knillmann, S., Schäfer, R.B., Liess, H.-D., 2016. Predicting the synergy of multiple stress effects. Sci. Rep. 6, 32965. https://doi.org/10.1038/srep32965.
Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30. https://doi.org/10.1016/j.aquatox.2010.10.006.
Lydy, M.J.J., Belden, J.B.B., Ternes, M.A.A., 1999. Environmental contamination and toxicology effects of temperature on the toxicity of M-parathion, chlorpyrifos, and pentachlorobenzene to Chironomus tentans. Arch. Environ. Contam. Toxicol. 547, 542–547.
Maazouzi, C., Coureau, C., Piscart, C., Saplairoles, M., Baran, N., Marmonier, P., 2016. Individual and joint toxicity of the herbicide S-metolachlor and a metabolite, deethylatrazine on aquatic crustaceans: Difference between ecological groups. Chemosphere 165, 118–125. https://doi.org/10.1016/j.chemosphere.2016.09.030.
Marigoudar, S.R., Mohan, D., Nagarjuna, A., Karthikeyan, P., 2018. Biomarker and histopathological responses of Lates calcarifer on exposure to sub lethal concentrations of chlorpyrifos. Ecotoxicol. Environ. Saf. 148, 327–335. https://doi.org/10.1016/j.ecoenv.2017.10.026.
Marsh, J.B., Weinstein, D.B., 1966. Simple charring method for determination of lipids. J. Lipid Res. 7, 574–576.
Miyamoto, S., Almeida, E.A., Nogueira, L., Gennari de Medeiros, M.H., Di Mascio, P., 2011. Evaluation of Malondialdehyde Levels: Oxidative Stress in Aquatic Ecosystems. John Wiley & Sons, Chichester, UK.
Moe, S.J., De Schamphelaere, K., Clements, W.H., Sorensen, M.T., Van den Brink, P.J., Liess, M., 2013. Combined and interactive effects of global climate change and toxicants on populations and communities. Environ. Toxicol. Chem. 32, 49–61. https://doi.org/10.1002/etc.2045.
Noyes, P.D., Lema, S.C., 2015. Forecasting the impacts of chemical pollution and climate change interactions on the health of wildlife. Curr. Zool. 61, 669–689. https://doi.org/10.1093/czoolo/61.4.669.
Op de Beeck, L., Janssens, L., Stoks, R., De Lin Op, B., Lizanne, J., Robby, S., 2016. Synthetic predator cues impair immune function and make the biological pesticide Bti more lethal for vector mosquitoes. Ecol. Appl. 26, 355–366. https://doi.org/10.1890/15-0326.
Op de Beeck, L., Verheyen, J., Olsen, K., Stoks, R., 2017a. Negative effects of pesticides under global warming can be counteracted by a higher degradation rate and thermal adaptation. J. Appl. Ecol. 54, 1847–1855. https://doi.org/10.1111/1365-2664.12919.
Op de Beeck, L., Verheyen, J., Stoks, R., 2017b. Integrating both interaction pathways between warming and pesticide exposure on upper thermal tolerance in high- and low-latitude populations of an aquatic insect. Environ. Pollut. 224, 714–721. https://doi.org/10.1016/j.envpol.2016.11.014.
Oracz, K., Bouteau, H.E.M., Farrant, J.M., Cooper, K., Belghazi, M., Job, C., Job, D., Corbineau, F., Bailly, C., 2007. ROS production and protein oxidation as a novel mechanism for seed dormancy alleviation. Plant J. 50, 452–465. https://doi.org/10.1111/j.1365-313X.2007.03063.x.
Pacheco, A., Martins, A., Guilhermino, L., 2018. Toxicological interactions induced by chronic exposure to gold nanoparticles and microplastics mixtures in Daphnia magna. Sci. Total Environ. 628–629, 474–483. https://doi.org/10.1016/j.scitotenv.2018.02.081.
Patetsini, E., Dimitriadis, V.K., Kaloyianni, M., 2013. Biomarkers in marine mussels, *Mytilus galloprovincialis*, exposed to environmentally relevant levels of the pesticides, chlorpyrifos and penoxsulam. Aquat. Toxicol. 126, 338–345. https://doi.org/10.1016/j.aquatox.2012.09.009.
Patra, R.W., Chapman, J.C., Lim, R.P., Gehrke, P.C., Sunderam, R.M., 2015. Interactions between water temperature and contaminant toxicity to freshwater fish. Environ. Toxicol. Chem. 34, 1809–1817. https://doi.org/10.1002/etc.2990.
Perschbacher, P.W., 2005. Temperature effects on acute copper toxicity to juvenile channel catfish Ictalurus punctatus. Aquaculture 243, 225–228.
Pestana, J.L.T., Loureiro, S., Baird, D.J., Soares, A.M.V.M., 2009. Fear and loathing in the benthos: responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk. Aquat. Toxicol. 93, 138–149. https://doi.org/10.1016/j.aquatox.2009.04.008.
Philippe, C., Grégoir, A.F., Thoré, E.S.J., Brendonck, L., De Boeck, G., Pinceel, T., 2018. Acute sensitivity of the killifish *Nothobranchius furzeri* to a combination of temperature and reference toxicants (cadmium, chlorpyrifos and 3,4-dichloroaniline). Environ. Sci. Pollut. Res. 25, 10029–10038. https://doi.org/10.1007/s11356-018-1278-x.
Core Team R, 2017. R: a language and environment for statistical computing. R Found. Stat. Comput https://doi.org/ISBN 3-900051-07-0.
Rubach, M.N., Baird, D.J., Boerwinkel, M.C., Maund, S.J., Roessink, I., Van Den Brink, P.J., 2012. Species traits as predictors for intrinsic sensitivity of aquatic invertebrates to the insecticide chlorpyrifos. Ecotoxicology 21, 2088–2101. https://doi.org/10.1007/s10646-012-0962-8.
Schäfer, R.B., Piggott, J.J., 2018. Advancing understanding and prediction in multiple stressor research through a mechanistic basis for null models. Glob. Change Biol. 1–10. https://doi.org/10.1111/gcb.14073.
Scheil, V., Köhler, H.-R., 2009. Influence of nickel chloride, chlorpyrifos, and imidacloprid in combination with different temperatures on the embryogenesis of the zebrafish Danio rerio. Arch. Environ. Contam. Toxicol. 56, 238–243.
Seeland, A., Albrand, J., Oehlmann, J., Müller, R., 2013. Life stage-specific effects of the fungicide pyrimethanil and temperature on the snail *Physella acuta* (Draparnaud, 1805) disclose the pitfalls for the aquatic risk assessment under global climate change. Environ. Pollut. 174, 1–9. https://doi.org/10.1016/j.envpol.2012.10.020.
Shin, S.M., Akram, W., Lee, J.J., 2012. Effect of body size on energy reserves in *Culex pipiens pallens* females (Diptera: culicidae). Entomol. Res. 42, 163–167. https://doi.org/10.1111/j.1748-5967.2012.00448.x.
Sinclair, B.J., Marshall, K.E., Sewell, M.A., Levesque, D.L., Willett, C.S., Slotsbo, S., Dong, Y., Harley, C.D.G., Marshall, D.J., Helmuth, B.S., Huey, R.B., 2016. Can we predict ectotherm responses to climate change using thermal performance curves and body temperatures? Ecol. Lett. 19, 1372–1385. https://doi.org/10.1111/ele.12686.
Singmann, H., Bolker, B., Westfall, J., Aust, F., 2017. Afex: Analysis of Factorial Experiments.
Sohal, R.S., Weindruch, R., 1996. Oxidative stress, caloric restriction, and aging. Science 273, 59–63.
Tran, T.T., Janssens, L., Dinh, K.V., Op de Beeck, L., Stoks, R., 2016. Evolution determines how global warming and pesticide exposure will shape predator-prey interactions with vector mosquitoes. Evol. Appl. 9, 818–830. https://doi.org/10.1111/eva.12390.
Tran, T.T., Janssens, L., Dinh, K.V., Stoks, R., 2018. Transgenerational interactions between pesticide exposure and warming in a vector mosquito. Evol. Appl. 1–12. https://doi.org/10.1111/eva.12605.
Tu, X.H., Zhuo, Y.L., Jiang, X.Y., Liu, H.L., Cao, Y., Liu, S., 2018. Molecular characterization of a mitochondrial manganese superoxide dismutase from *Chilo suppressalis* (Lepidoptera: crambidae). J. Econ. Entomol. 111, 2391–2400. https://doi.org/10.1093/jee/toy180.
Van den Brink, P.J., Boxall, A.B.A., Maltby, L., Brooks, B.W., Rudd, M.A., Backhaus, T., Spurgeon, D., Verougstraete, V., Ajao, C., Ankley, G.T., Apitz, S.E., Arnold, K., Brodin, T., Cañedo-Argüelles, M., Chapman, J., Corrales, J., Coutellec, M.A., Fernandes, T.F., Fick, J., Ford, A.T., Giménez Papiol, G., Groh, K.J., Hutchinson, T.H., Kruger, H., Kukkonen, J.V.K., Loutseti, S., Marshall, S., Muir, D., Ortiz-Santaliestra, M.E., Paul, K.B., Rico, A., Rodea-Palomares, I., Römbke, J., Rydberg, T., Segner, H., Smit, M., van Gestel, C.A.M., Vighi, M., Werner, I., Zimmer, E.I., van Wensem, J., 2018. Toward sustainable environmental quality: priority research questions for Europe. Environ. Toxicol. Chem. 37, 2281–2295. https://doi.org/10.1002/etc.4205.
Vellinger, C., Felten, V., Rousselle, P., Beisel, J.-N., Sornom, P., Usseglio-Polatera, P., 2012. Behavioural and physiological responses of *Gammarus pulex* exposed to cadmium and arsenate at three temperatures: individual and combined effects. PLoS One 7https://doi.org/10.1371/journal.pone.0039153. e39153.
Vinebrooke, R.D., Cottingham, K.L., Norberg, J., Scheffer, M., Dodson, S.I., Maberly, S.C., Sommer, U., 2004. Impacts of multiple stressors on biodiversity and ecosystem functioning: the role of species co-tolerance. Oikos 104, 451–457.
Vrzal, E.M., Allan, S.A., Hahn, D.A., 2010. Amino acids in nectar enhance longevity of female *Culex quinquefasciatus* mosquitoes. J. Insect Physiol. 56, 1659–1664. https://doi.org/10.1016/j.jinsphys.2010.06.011.
Weis, J.S., Mantel, L.H., 1976. DDT as an accelerator of limb regeneration and molting in fiddler crabs. Estuar. Coast. Mar. Sci. 4, 461–466. https://doi.org/10.1016/0302-3524(76)90020-7.
WHO, 2005. Guidelines for laboratory and field testing of mosquito larvicides. World Heal. Organ. 1–41 https://doi.org/Ref: WHO/CDS/ WHOPES/GCDPP/2005.11.
Yu, S., Wages, M., Willming, M., Cobb, G.P., Maul, J.D., 2015. Joint effects of pesticides and ultraviolet-B radiation on amphibian larvae. Environ. Pollut. 207, 248–255. https://doi.org/10.1016/j.envpol.2015.09.029.
Zahran, E., Risha, E., Awadin, W., Palić, D., 2018. Acute exposure to chlorpyrifos induces reversible changes in health parameters of Nile tilapia (*Oreochromis niloticus*). Aquat. Toxicol. 197, 47–59. https://doi.org/10.1016/j.aquatox.2018.02.001.